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Dilkamural: A novel chemical weapon involved in the invasive capacity of the alga *Rugulopteryx okamurae* in the Strait of Gibraltar

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ABSTRACT

The southwestern coasts of Europe (Strait of Gibraltar) are experiencing a severe invasion of the brown alga *Rugulopteryx okamurae*, original from the northwestern Pacific ocean. Currently there is no clue regarding to the reasons of such huge invasive potential, although the involvement of chemical defenses has recently been suggested. In this context, this study was aimed to investigate the presence and potential role of chemical defenses in the invasive success of *R. okamurae*. The chemical study of *R. okamurae* from the Strait of Gibraltar led to the isolation of six secondary metabolites, among which the compound dilkamural stands out because of its high concentration. Later, in a set of feeding deterrent assays, the generalist native herbivore *Paracentrotus lividus* showed higher consumption over the native alga *Ulva* sp. than over the non-native *R. okamurae*. This low consumption was tracked down to dilkamural, which displayed not only deterrent properties but also caused harmful and even lethal effects over the sea urchins. These results are consistent with the novel weapons hypothesis, since dilkamural was not described previously in the invaded area and has a defensive role against generalist herbivores in the new range, thus helping to explain the great expansion of *R. okamurae* in the Strait of Gibraltar.

1. Introduction

In the last decades, the rate of non-indigenous marine species entering new ranges has experienced an unprecedented increase, mainly due to the continuous expansion of shipping transport, aquaculture, and aquarium trade (Bax et al., 2003; Katsanevakis et al., 2013; Galil et al., 2018). Macroalgae, which have a significant contribution to the number of alien species (Schaffelke et al., 2006; Williams and Smith, 2007), are a worrying group, since some introduced species have shown capability to successfully spread and reach densities much higher than in their native regions, causing significant ecological and economic impacts on the recipient communities (Piazzi and Ceccherelli, 2006; G. Schaffelke and Hewitt, 2007; Martinez et al., 2012).

The European coasts have been affected by several macroalgal invasions, among which those of the green alga *Caulerpa cylindracea* (formerly *C. racemosa* var. *cylindracea*) throughout the Mediterranean coasts, and of the brown alga *Sargassum muticum* throughout the Atlantic coasts, have been especially severe (Klein and Velarque, 2008; Engelen et al., 2015). Currently, a new episode of macroalgal invasion is taking place in the southwestern European coasts, at the Strait of Gibraltar, where the brown alga *Rugulopteryx okamurae* is dramatically spreading (Navarro-Barranco et al., 2019; García-Gómez et al., 2020). The species *R. okamurae* is widely distributed in the northwestern Pacific ocean, throughout the coasts of China, Japan, Korea, Philippines, and Taiwan (Agatsuma et al., 2005). This alga was firstly described as *Dilophus okamurae* (Dawson, 1950), but ten years ago it was transferred to the genus *Rugulopteryx* (Hwang et al., 2009). *R. okamurae* was observed for the first time in European waters in 2002, likely introduced in the Mediterranean coasts of France with Japanese oysters used for aquaculture (Velarque et al., 2009). Since 2016–2017, *R. okamurae* has experienced a massive proliferation and continuous spread along the coasts of the Strait of Gibraltar, which connects the Mediterranean sea and the Atlantic ocean (El Aamri et al., 2018; Navarro-Barranco et al., 2019). This is a marine protected area inhabited by a great variety of seaweeds, which are progressively being displaced by *R. okamurae* (Navarro-Barranco et al., 2019; García-Gómez et al., 2020). Moreover, this alga has become a dominant species that fouls fishing gear and nets and throws tons of beach cast material on touristic beaches, thus beating on two economic cornerstones of the region.

Different abiotic and biotic factors have been shown to be involved

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either in the failure or in the successful establishment of invasive macroalgae (Occhipinti-Ambrogi and Savini, 2003; Piazzi et al., 2016; Cardeccia et al., 2018; Geburzi and McCarthy, 2018). Among such factors, there is growing consensus about the crucial role that the chemical defenses (i.e. secondary metabolites, also known as natural products) produced by the invader may play (Lages et al., 2015; Mollo et al., 2015; Máximo et al., 2018). It is well established that marine organisms have developed an array of mechanisms, including chemical strategies, to ensure fitness and survival. Chemical strategies rely on the production or accumulation of bioactive compounds finely tuned to play key roles in defense against predation and pathogen attack, mediation of spatial competition, and facilitation of reproduction, among others (Harper et al., 2001; Puglisi et al., 2014). As a result, these metabolites exert crucial effects on the relationships between organisms, species distribution, and community organization, as well as influence feeding patterns and the selection for traits contributing to maintenance of biodiversity (Hay 2009, 2014). On this basis, it has been proposed that when a species reaches a new range, its bioactive secondary metabolites may cause unexpected and dramatic effects on a recipient community that has never been exposed to those compounds (Mollo et al., 2015).

In this regard, chemical studies of native specimens of *R. okamurae* collected at several locations of the Japanese coasts showed the presence of an array of secondary metabolites of the terpenoid class (Ochi et al., 1982; Kurata et al., 1988a, 1988b, 1989, 1990, 1988b, 1990; Ninomiya et al., 1999; Yamase et al., 1999; Suzuki et al., 2002). Interestingly, some of these compounds were reported to inhibit the settlement and metamorphosis of the larvae of the abalone *Haliotis discus hannai* Ino (Kurata et al., 1988b) and to possess feeding deterrent activity against the young abalone (Kurata et al., 1988a, 1989, 1990, 1989; Suzuki et al., 2002) and against young sea urchin *Strongylocentrotus nudus* (Kurata et al., 1990), thus suggesting that the natural products of *R. okamurae* could play a chemical defense function. Currently, there are neither data on the secondary metabolites of the invasive specimens of *R. okamurae* that grow in the southwestern European coasts, nor studies on the potential role of these compounds in the success of its invasive behavior that could contribute to explain how this species shifted from exotic to invasive in such short period of time (Navarro-Barranco et al., 2019; García-Gómez et al., 2020).

In particular, the knowledge of the activity of the secondary metabolites against herbivores is of utmost interest, since the impact of herbivory may be a decisive factor for the success of macroalgal invasions (Máximo et al., 2018). In this regard, the enemy release hypothesis (ERH) proposes that in the new colonized area, introduced species are free from their specialist enemies (e.g. herbivores and pathogens) and, therefore, they should experience a limited negative impact since local enemies disregard them as suitable food or hosts (Keane and Crawley, 2002). However, new areas may be free of specialized enemies but plenty of generalist herbivores that may actively graze on the invasive species and therefore increase the biotic resistance of the area (Elton, 1958; Morrison and Hay, 2011). Consequently, the ability of invasive species to escape from predation by native generalist herbivores may enhance the likely of invasion success, as stated by the shifting defense hypothesis (SDH). This hypothesis proposes that the selection in invasive plants may not only lead to the evolution of reduced defenses against specialist herbivores, but also to the evolution of higher levels of defense against generalist herbivores (Müller-Schärer et al., 2004; Joshi and Vrieling, 2005; Zhang et al., 2018). The later can be accomplished by increasing resistance strategies (i.e. physical and chemical mechanisms) as, for instance, the production of high-concentrations of digestibility-reducers and/or toxins (Müller-Schärer et al., 2004; Joshi and Vrieling, 2005).

The present study was aimed to analyze the role of the chemical defenses in the alga *R. okamurae* that is quickly spreading throughout the southwestern European coasts, in order to find clues regarding to its large invasive potential. We hypothesized that the alga *R. okamurae* which invades the Strait of Gibraltar may possess a wide arsenal of bioactive metabolites (e.g. those described by Ochi et al., 1982; Kurata et al., 1988a, 1988b, 1989, 1990; Ninomiya et al., 1999; Yamase et al., 1999; Suzuki et al., 2002) with deterrent properties against generalist herbivores of the invaded area, but that these compounds are different from those produced by native macroalgal species (Durán et al., 1997; De Paula et al., 2011; De los Reyes et al., 2016), as stated by the novel weapons hypothesis. In addition, if the higher invasive potential of this species is related to the selection of those genotypes of *R. okamurae* bearing higher chemical defenses, as stated by the shifting defense hypothesis, we should find both higher concentrations than those recorded in their native area and a concentration dependence of the deterrent activity (Joshi and Vrieling, 2005; Doorduin and Vrieling, 2011). Therefore, a laboratory experimental approach was used (1) to identify the secondary metabolites of the invasive alga *R. okamurae*, (2) to examine the feeding behaviour of a generalist herbivore from the invaded area (i.e. *Paracentrotus lividus)* on the invasive alga, and (3) to investigate the effects of the major metabolite isolated from the invasive alga on sea urchins of the invaded area.

2. Materials and methods

2.1. Isolation and identification of secondary metabolites from Rugulopteryx okamurae of the Strait of Gibraltar

Samples of *R. okamurae* were collected in Punta Carnero (Cádiz, Spain) (36°04′38.6″N; 5°25′31.1″ W) in November. After washing with fresh water to remove epiphytes and organic and inorganic debris, algae were frozen at −20 °C until the extraction procedure.

2.1.1. Extraction of R. okamurae

A mixture of acetone/methanol (MeOH) (143 mL, 1:1, v/v) was added to frozen algae (10.2 g dry weight after extraction), the mixture was mashed for 5 min and subjected to sonication for another 8 min. The solution was filtered over paper and the residual algal material extracted six more times following the same procedure. The solutions were combined and the organic solvent evaporated under reduced pressure. The remaining aqueous residue was extracted with diethyl ether (Et_2O) (3 × 75 mL). The organic layers were combined, dried over anhydrous $MgSO_4$, and the solvent evaporated in a rotary evaporator to yield a dark green oily extract (1.55 g in total).

2.1.2. Purification and identification of secondary metabolites

The extract of *R. okamurae* was separated into fractions from A to I by column chromatography on silica gel (60–200 μm) using mixtures of *n*-hexane (Hex) and diethyl ether (Et_2O) of increasing polarity (Hex/Et_2O, v/v, 90:10, 175 mL; 80:20, 200 mL; 70:30, 200 mL; 50:50, 400 mL; 30:70, 300 mL), then Et_2O (200 mL), and finally mixtures of chloroform/methanol ($CHCl_3$/MeOH, v/v, 90:10, 200 mL; 80:20, 200 mL; 50:50, 100 mL). The fraction F (512.4 mg), which contained the major natural product of the extract, was purified on two SPE-C18 cartridges (1 g/6 mL) eluted with 10 mL each of MeOH/H_2O 95:5 (v/v). The resulting mixture was subjected to normal phase HPLC (Kromasil 100-5SIL column, 250 × 10 mm, 5 μm) using Hex/ethyl acetate (EtOAc) 75:25 (v/v) as eluent, yielding the major natural product of the extract (compound **1**) together with minor amounts of another product (compound **2**). Similarly, the fraction G (208.7 mg) was separated on a SPE-C18 cartridge using MeOH/H_2O 9:1 (10 mL) and then by reversed phase HPLC (Kromasil 100-5C18 column, 250 × 10 mm, 5 μm) eluted with MeOH/H_2O 8:2, yielding a pure product (compound **3**). Fractions B (65.6 mg) and C (41.1 mg) were separated by normal phase HPLC using Hex/EtOAc 95:5 (v/v) and Hex/EtOAc 90:10 (v/v) as eluents, respectively, yielding another three pure compounds (compound **4** from fraction C and compounds **5** and **6** from fraction B). The isolated compounds were identified by Nuclear Magnetic Resonance (NMR) (Agilent-500 spectrometer) (Appendix A) and comparison with literature data (Kurata et al., 1988a, 1988b, 1990, 1988b; Ninomiya et al., 1999; Yamase et al.,

1999).

2.2. Quantitative analysis of the major secondary metabolite in Rugulopteryx okamurae

To define the amount of the major metabolite (compound **1**) of *R. okamurae* to be used in the feeding assays, the mean concentration of this compound in six samples of fresh algae was determined by quantitative analysis of the corresponding extracts. Each algal sample (approx. 10–11 g fresh weight) was mixed with 15 mL of acetone/MeOH (1:1, v/v), mashed, and subjected to sonication for 5 min. The solution was filtered over paper and the residual algal material was extracted five more times using the same procedure. The solutions were combined and evaporated to dryness at reduced pressure to yield dark green extracts. The content of compound **1** in each extract was determined by quantitative ^{1}H-NMR, using 1,3,5-trimethoxybenzene (99% purity, Sigma-Aldrich TraceCERT) as internal standard (IS) and CD_3OD as solvent. The ^{1}H-NMR spectra were recorded on a 500 MHz Agilent spectrometer using the following parameters: spectral width = 8992.8 Hz, pulse width = 8.1 μs (90°), relaxation delay = 30.0 s, acquisition time = 3.0 s, and lb = 0.3 Hz for processing. The aldehyde proton signal of compound **1** (δ_H 9.61, 1H) and the aromatic protons signal of the IS (δ_H 6.07, 3H) were used for quantification. The start and end points for integration of each peak were selected automatically. Once determined the amount of compound **1** in the extract, this datum together with the dry weight of the extracted alga allowed to determine the total content of compound **1** with respect to algal dry weight. The content of compound **1** in fresh alga was determined by taking into account that for the examined samples 1.0 g of fresh alga yielded 0.177 ± 0.009 (n = 6) g of dry weight.

2.3. Set-up of the feeding-deterrent assays

2.3.1. Collection of sea urchins

The generalist herbivore *Paracentrotus lividus* (purple sea urchin) was collected from a rocky shore, La Caleta, in Cádiz, Spain (36°31′39″N; 6°18′46″W), where an abundant and stable population inhabits. Once permission was granted by local environmental authorities, 150 individuals were collected at a depth of 2 m. Harvesting was carefully carried out by snorkeling, avoiding damage to the organisms. Only individuals with sizes between 3 and 5 cm in diameter (adult size) were gathered. Collected organisms were kept in cooled containers with seawater and transported to the laboratory, where they were haphazardly placed in aerated tanks with running seawater at a temperature of 18 °C. Sea urchins were fed with *Ulva* sp. for 4 days until the beginning of the experiment, allowing their acclimation to experimental conditions (Vergés et al., 2007). During this time, the photoperiod was set at 8:16 h (light:darkness) because *P. lividus* usually exhibits nocturnal activity (Boudouresque and Verlaque, 2001).

2.3.2. Collection of algae

Specimens of the green alga *Ulva* sp. were collected in July, in the intertidal zone of the internal bay of Cadiz, (36°27′57.3″N; 6°14′49.7″W), cleaned with seawater and transported to the laboratory in a cooled container with seawater. A portion of fresh biomass (100 g) was freeze-dried and the remaining material (*ca.* 500 g) was maintained in an aquarium with seawater and continuous aeration, until used as feed for the sea urchins and in the feeding-deterrent assays. The brown alga *R. okamurae* was collected in July in the intertidal zone of the coasts of Tarifa (Cádiz, Spain) (36°00′43.5″N; 5°35′50.3″ W), cleaned with seawater and transported to the laboratory in a cooled container with seawater. A portion of the biomass was freeze-dried, another portion was used for the quantitative analysis of the major metabolite previously identified (compound **1**), and the remaining material was kept in an aquarium with seawater and continuous aeration until the feeding-deterrent assays.

2.3.3. Feeding-deterrent assays

The experiments were carried out in aquaria (24 aquaria in total) with natural seawater in a closed flow-through system (10 L in each aquarium), located in a chamber with controlled light and temperature (18 °C). The aquaria were illuminated (approx. 100 μmol photons m^{-2} s^{-1} in the bottom of the aquaria) with cool fluorescent tubes (T5 High Output Blau Aquaristic aquarium color extreme fluorescents) in a 8:16 h (light:darkness) photoperiod.

Prior to the feeding-deterrent assays, the sea urchins were starved for 24 h. In each aquarium, three sea urchins of the same size randomly collected from the starved pool, were placed with one of the six following diets: i) fresh *Ulva* sp. (U_f), ii) fresh *R. okamurae* (R_f), iii) agar blocks containing freeze-dried *Ulva* sp. (U_{fd}), iv) agar blocks containing a 1:1 mixture of freeze-dried *Ulva* sp. and freeze-dried *R. okamurae* (U_{fd} + L_{fd}), v) agar blocks containing freeze-dried *Ulva* sp. coated with the major metabolite extracted from *R. okamurae* (compound **1**) at the natural concentration measured in fresh biomass (U_{fd} + DK_N), vi) agar blocks containing freeze-dried *Ulva* sp. coated with the major metabolite extracted from *R. okamurae* at 25% of the concentration found in fresh biomass (U_{fd} + DK_L).

In all cases, 6 g of food were supplied to each aquarium in order to keep a constant ratio between available food and sea urchins. Fresh *Ulva* sp. and fresh *R. okamurae* were submerged but free floating in the aquaria and water level was low enough to make the algae available to the sea urchins. The agar block (6 g each) was cut into four portions in order to facilitate the access of the animals to the food. Then, these four agar portions were randomly allocated in the bottom of the aquarium. Agar blocks were prepared at a concentration of 2% (weight/volume) with distilled water and agar, and the amount of freeze-dried biomass corresponding to 6 g in total of fresh material (i.e. U_{fd}, R_{fd} and U_{fd} + R_{fd} diets) was also included in the mixture. The last two diets (i.e. U_{fd} + DK_N and U_{fd} + DK_L) were aimed to test the deterrent activity of the major compound extracted from *R. okamurae* (compound **1**). In order to test this compound at the mean concentration found in the invasive algae, the amount of compound corresponding to 6 g of fresh *R. okamurae* (i.e. 45 mg of product) was dissolved in diethyl ether (2 mL), mixed with lyophilized *Ulva* sp. obtained from freeze-drying 6 g of fresh algae and then the solvent was allowed to evaporate at room temperature (Vergés et al., 2007). The resulting freeze-dried *Ulva* sp. coated with the major natural product of *R. okamurae* was employed to prepare the agar-based diet U_{fd} + DK_N following the same aforementioned procedure. The diet U_{fd} + DK_L was prepared in a similar way to the previous diet, but using a lower concentration of the compound (25%). The palatable *Ulva* sp. was included in these diets in order to encourage the feeding of sea urchins over the artificial diets (Lyons et al., 2007). Preliminary feeding assays performed with *Ulva* sp. treated with diethyl ether allowed to confirm that the addition and subsequent evaporation of the solvent did not cause toxicity and did not affect to the consumption of agar blocks by sea urchins (data not shown).

Autogenic controls (i.e. agar-based diets placed in an aquarium but without *P. lividus*) under the same experimental conditions were also performed, to account for potential changes in agar blocks weight not due to grazing. Results showed no significant changes in the weight of the autogenic agar blocks in any of the assays and, therefore, were not further considered in the analysis (Student's *t*-test: t (U_f) = −0.36, df = 2, $p > 0.05$, t (R_f) = 1.56, df = 2, $p > 0.05$, t (U_{fd}) = 4.50, df = 2, $p > 0.05$, t (U_{fd} + R_{fd}) = −1.18, df = 2, $p > 0.05$, t (U_{fd} + DK_N) = −1.18, df = 2, $p > 0.05$, t (U_{fd} + DK_L) = −3.35, df = 2, $p > 0.05$).

The diets (fresh algae or agar blocks) were weighed at the beginning of the assay and after 24 h of experimental time. Consumption rates (CR) were calculated as follows:

$$CR = \frac{Wo - Wf}{\Delta T\, N}$$

where W_0 and W_f are the initial and final fresh weights of algae or agar

blocks, ΔT is the lapsed time in days and N is the number of experimental sea urchins in the aquarium. Feeding rate is finally expressed as g FW individual^{-1} day^{-1}. At the end of the experiment, the physiological state of each sea urchin in each aquarium was analyzed and classified in one of the following categories: i) alive and healthy, ii) alive but exhibiting approx. 25% of spines loss, iii) alive but exhibiting approx. 75% of spines loss and iv) dead (when all spines were lost), and was finally expressed as the % of sea urchins in each one of the aforementioned categories. Four replicates were used for each diet.

2.4. Statistical analysis

All data were tested for normality (Shapiro-Wilk normality test) and homoscedasticity (Barlett test of homogeneity of variances test) prior to the analyses. Feeding rates were square root-transformed to satisfy the assumption of homogeneity of variance. A one-way ANOVA and Tukey's post hoc analyses were applied to assess significant differences in the feeding assays. Data are presented as mean ± SE.

3. Results

3.1. Secondary metabolites from R. okamurae of the Strait of Gibraltar

The chemical study of *R. okamurae* collected in the coasts of the Strait of Gibraltar led to obtain six secondary metabolites (compounds **1**–**6**) synthetized by the alga. The spectroscopic analysis, mainly performed by NMR (Appendix A), allowed the identification of compounds **1**–**6** as diterpenes whose chemical structures are shown in Fig. 1. The major metabolite was compound **1**, known as dilkamural, which represented 28.25% (w/w) of the diethyl ether extract. The remaining isolated compounds **2**–**6** were one or even two orders of magnitude less abundant, and represented 3.17%, 0.23%, 0.21%, 0.17%, and 1.55% (w/w) of the extract, respectively. On the other hand, the quantitative ^{1}H NMR analysis of the acetone/methanol extracts obtained from fresh algae (n = 6), allowed to determine that the mean content of dilkamural was 4.21 ± 0.39% of dry weight of algae.

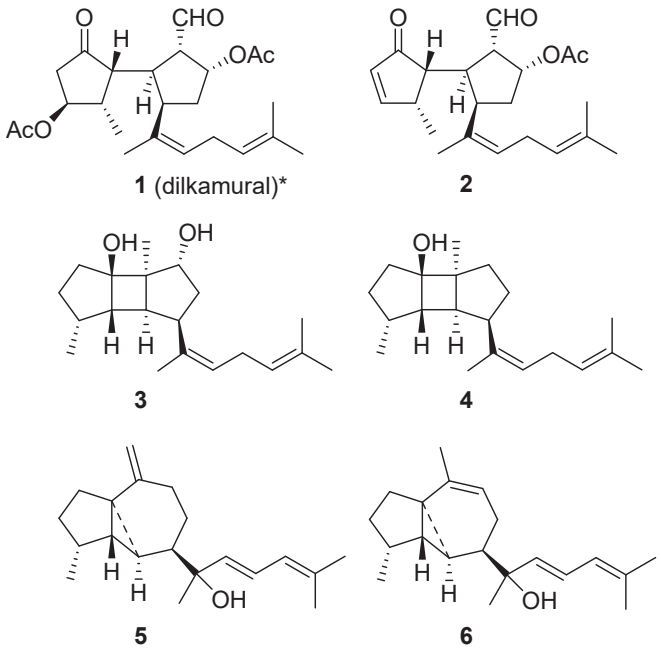

Fig. 1. Chemical structures of the six secondary metabolites (compounds **1**–**6**) isolated from *R. okamurae* collected at the Strait of Gibraltar. The major compound is marked with an asterisk.

3.2. Feeding-deterrent assays

Among fresh algae, *Ulva* sp. (U_f) was the preferred food for sea urchins (consumption of 0.947 ± 0.111 g FW individual^{-1} day^{-1}) while the consumption rate over *R. okamurae* (R_f) was significantly lower (0.257 ± 0.053 g FW individual^{-1} day^{-1}; MS = 0.926, F = 47.65, df = 5, $p < 0.001$) (Fig. 2). The agar-based diet containing freeze-dried *Ulva* sp. (U_{fd}) was much more consumed than the agar diet containing a 1:1 mixture of freeze-dried *Ulva* sp. and freeze-dried *R. okamurae* ($U_{fd} + R_{fd}$) (Fig. 2). When the major metabolite of *R. okamurae* (i.e. dilkamural) was included in the diet at the mean concentration measured in fresh algae from their non-native range ($U_{fd} + DK_N$), a large drop in the consumption rate was recorded, reaching values near to zero (Fig. 2). However, when the concentration of dilkamural was reduced to 25% of that found in fresh algae ($U_{fd} + DK_L$) the consumption rate increased to intermediate values between freeze-dried *Ulva* (U_{fd}) and freeze-dried *Ulva* sp. plus freeze-dried *R. okamurae* ($U_{fd} + R_{fd}$) (Fig. 2).

3.3. Physiological state of sea urchins after feeding-deterrent assays

Diets containing invasive *R. okamurae* (fresh and freeze-dried) or its major secondary metabolite (dilkamural) did not only reduce the consumption rates of *P. lividus*, but also greatly affected the physiological state of the sea urchins, recording even lethal effects in 24 h (Fig. 3).

While all sea urchins in the aquaria with fresh or freeze-died *Ulva* sp. (i.e. diets U_f and U_{fd}) or with dilkamural at a concentration 25% of that recorded in fresh *R. okamurae* (i.e. diet $U_{fd} + DK_L$) were healthy after 24 h, a large proportion (67%) of the animals in the aquaria with fresh *R. okamurae* (diet R_f) died during the course of the experiment, and the remaining, although alive, were greatly affected with most of them having lost up to 75% of their spines. When animals were fed with a mixture of freeze-dried *Ulva* sp. and freeze-dried *R. okamurae* (diet $U_{fd} + R_{fd}$) mortality and harmful effects were also observed. Moreover, the mortality was increased up to 58% among those sea urchins fed with agar blocks containing dilkamural at the concentration recorded in fresh *R. okamurae* (diet $U_{fd} + DK_N$) and another 33% of the animals displayed important detrimental effects, as indicated by the loss of up 75% of their spines (Fig. 3).

4. Discussion

Native herbivores have a large potential to control invasive macroalgal species (e.g. a part of the biotic resistance hypothesis; Elton, 1958;

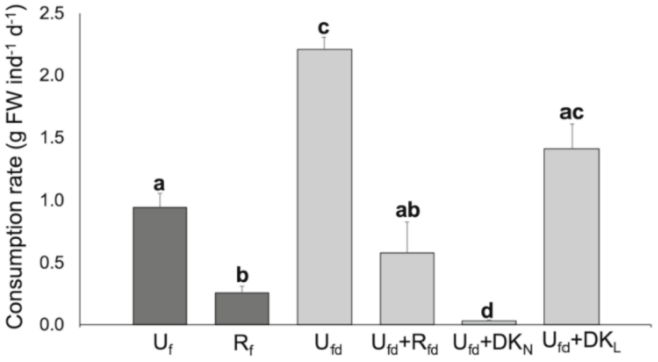

Fig. 2. Feeding rates of *P. lividus* over experimental diets: U_f = fresh *Ulva* sp.; R_f = fresh *R. okamurae*, U_{fd} = agar blocks containing freeze-dried *Ulva* sp.; $U_{fd} + R_{fd}$ = agar blocks containing a 1:1 mixture of freeze-dried *Ulva* sp. and freeze-dried *R. okamurae*; $U_{fd} + DK_N$ = agar blocks containing freeze-dried *Ulva* sp. coated with dilkamural at the mean concentration recorded in fresh *R. okamurae*; $U_{fd} + DK_L$ = agar blocks containing freeze-dried *Ulva* sp. coated with dilkamural at 25% of the mean concentration recorded in fresh *R. okamurae*. Data are shown as mean ± SE (n = 4). Bold letters indicate significant differences at $p < 0.05$.

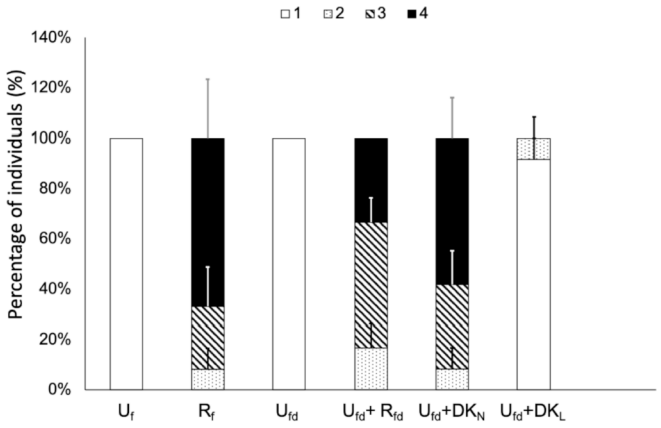

Fig. 3. Physiological state of *P. lividus* at the end of the feeding-deterrent assays. The diets in the experiment were: U_f = fresh *Ulva* sp.; R_f = fresh *R. okamurae*, U_{fd} = agar blocks containing freeze-dried *Ulva* sp.; $U_{fd} + R_{fd}$ = agar blocks containing a 1:1 mixture of freeze-dried *Ulva* sp. and freeze-dried *R. okamurae*; $U_{fd} + DK_N$ = agar blocks containing freeze-dried *Ulva* sp. coated with dilkamural at the mean concentration recorded in fresh *R. okamurae*; $U_{fd} + DK_L$ = agar blocks containing freeze-dried *Ulva* sp. coated with dilkamural at 25% of the mean concentration recorded in fresh *R. okamurae*. Inset numbers indicate the physiological state of the sea urchins at the end of the experiment: 1 = alive and healthy; 2 = alive but with 25% of spines loss; 3 = alive but with 75% of spines loss; 4 = dead. Data are shown as mean ± SE (n = 4).

Paul et al., 2001; Jormalainen and Honkanen, 2008; Morrison and Hay, 2011; Sotka et al., 2019). Nonetheless, when reaching non-native areas, macroalgae can be released from their specialist enemies (e.g. herbivores and pathogens) as stated by the enemy release hypothesis (Keane and Crawley, 2002), leaving generalist herbivores as the main barrier of the biotic resistance in the invaded area (Maron and Vilà, 2001; Cebrian et al., 2011; Tomas et al., 2011; Noè et al., 2018). This study has clearly demonstrated that the great invasive capacity of *Rugulopteryx okamurae* in the Gibraltar Strait (García-Gómez et al., 2020) could be favoured by the presence in this species of dilkamural (compound **1** in Fig. 1), a novel compound in the invaded area, or exotic chemical weapon *sensu* Callaway and Ridenour (2004), which makes *R. okamurae* not only less palatable for sea urchins, but also causes great toxic effects on this generalist herbivore in one day. In addition, the results of this study have highlighted that both, the deterrent and toxic capacities of this chemical weapon are concentration-dependent, since the compound was highly deterrent and toxic at the natural concentration found in algae collected in the invaded area (Strait of Gibraltar) while moderately deterrent and non toxic at lower concentrations.

Plants (including macroalgae) have evolved different strategies to face herbivory, which can be summarized in resistance *versus* tolerance strategies (Fineblum and Rausher, 1995; Pilson, 2000), although a mixture of defensive traits may be more realistic to understand the final outcome. Resistance strategy is thought to be relatively costly (Stamp, 2003; Zhang et al., 2018) and include physical defenses and/or the synthesis of bioactive compounds capable to deter herbivory by diminishing the palatability of algal tissues or even reduce herbivore fitness by causing noxious effects on the predator (Paul et al., 2001; Young et al., 2015; Zhang et al., 2018).

Previous chemical studies of *R. okamurae* (at the time known as *Dilophus okamurae*) performed on specimens from its native range (i.e. Japanese coasts; Agatsuma et al., 2005) showed the capacity of *R. okamurae* to produce an array of secondary metabolites of the diterpenoid class (Ochi et al., 1982; Kurata et al., 1988a, 1988b, 1989, 1990; Ninomiya et al., 1999; Yamase et al., 1999; Suzuki et al., 2002). Six of these metabolites (Fig. 1) have also been found in our study of invasive specimens collected in the coasts of the south of Spain (Strait of Gibraltar). Nonetheless, the particular set of compounds (Fig. 1) and the concentrations found in *R. okamurae* from the Strait of Gibraltar largely differ from those previously recorded from Japanese specimens. In particular, our chemical study has shown that a distinctive feature of the invasive *R. okamurae* is the high content of the compound dilkamural (4.21 ± 0.39% of algal dry weight), a natural product that had been found in a Japanese collection of *R. okamurae* (Ninomiya et al., 1999). Moreover, in spite of the wide chemical research performed on the natural products from algae around the world (Leal et al., 2013; Máximo et al., 2018; Carroll et al., 2019), a survey of the literature shows that so far dilkamural has only been described from the brown alga *R. okamurae* (Ninomiya et al., 1999). It is also worth noting that the chemical profile herein disclosed for the invasive alga is completely different from that described for the sympatric native macroalga *Dictyota dichotoma* (Durán et al., 1997; De Paula et al., 2011), which belongs to the same family (Dictyotaceae) and exhibits high morphological similarity to *R. okamurae* in the invaded area (Navarro-Barranco et al., 2019). These data suggest that dilkamural, which causes harmful effects on *P. lividus*, is evolutionary novel for native generalist herbivores of the Strait of Gibraltar. This finding is consistent with the novel weapons hypothesis (Callaway and Ridenour, 2004; Svensson et al., 2013), which is based on the main premise that the existence of a chemical compound in the invader (in this case dilkamural) is responsible of the low preference of native consumers for the invaders, providing them advantage for their expansion in the new community.

R. okamurae is known to be consumed by specialist and generalist mesograzers like sea urchins (*Strongylocentrotus nudus*) and abalones (*Haliotis discus hannai*) in its natural range (Agatsuma et al., 2005). The sea urchin *P. lividus* is a generalist herbivore widely distributed along the Mediterranean and northeastern Atlantic coasts, from Scotland to the south of Morocco (Boudouresque and Velarque, 2001) and has large capacity to regulate the distribution, abundance, and diversity of native and non-native seaweeds (Sumi and Scheibling, 2005; Monteiro et al., 2009; Cacabelos et al., 2010; Cebrian et al., 2011; Tomas et al., 2011; Cardoso et al., 2020). Therefore, this species is a potential predator and biological regulator of the invasive *R. okamurae* in the Strait of Gibraltar, as demonstrated for other macroalgal invaders. This study has shown that *P. lividus* consumes *R. okamurae* at a significantly lower rate than the native seaweed *Ulva* sp. Moreover, the feeding over *Ulva* sp. was not affected by the structural differences between algae, since *P. lividus* also exhibited higher consumption over blocks of agar that contain freeze-dried *Ulva* sp. over those with a mixture of *Ulva* sp. and *R. okamurae*. These results are in line with other studies that have reported the selective feeding capacity of *P. lividus*, which reduced its consumption rate over some invasive algae from the Mediterranean sea and Madeira island in comparison with native species (Cebrian et al., 2011; Tomas et al., 2011; Ramalhosa et al., 2016; Noè et al., 2018), and also with previous accounts recording the deterrent effects of the extracts of *R. okamurae* from its native area over young benthic herbivores like sea-urchins (*Strongylocentrotus nudus*) and abalones (*Haliotis discus hannai*) (Shiraishi et al., 1991).

In addition to the low consumption rates of diets containing fresh or freeze-dried *R. okamurae*, the sea urchins exhibited toxic and even lethal effects (Fig. 3) in a short period of time (24 h). Previous accounts on the toxicity of algae over *P. lividus* describe that feeding over *Caulerpa taxifolia* as the sole source of food caused physiological disorders in the sea urchins, including marked loss of spines, long righting times, small gonosomatic ratios, and mortality (Lemée et al., 1996; Bouduresque et al., 1996). These effects were evident at longer times than in our experiment (24 h *vs* several weeks) and were mainly attributed to the presence in *C. taxifolia* of toxic secondary metabolites (caulerpenyne) and/or to the low food intake by the sea urchins along the experiments. In the present study the low feeding of *P. lividus* over *R. okamurae* has been tracked down to a specific compound of this alga (dilkamural), as demonstrated by the lack of consumption and the strong toxic effects found in the diet containing pure dilkamural at the mean concentration recorded in the invasive specimens. The deterrent capacity of this

compound, together with the acute toxic effects observed in *P. lividus* may have significant consequences in the invasive capacity of *R. okamurae*, since maybe this microalga is not eaten at all in the invaded area. However, more studies are needed to disclose the underlying mechanism behind the acute toxicity exhibited by dilkamural against *P. lividus*, and whether both the deterrent capacity and toxic effects are extensive to other native herbivores.

The toxicity of dilkamural is concentration-dependent since mild deterrence, but not significant harmful effects, were observed on sea urchins whose diet contained a concentration of dilkamural lower than that found in the invasive specimens. These results suggest a potential relationship between the concentration of dilkamural and the invasiveness of *R. okamurae*, a fact that brings two likely explanations to delve in future research. First, as stated in the shifting defense hypothesis, the lack of specialist herbivores in the new area may reduce the cost of defense against such enemies and increase the levels of defense (e.g. toxins) against generalist herbivores of the new areas (Joshi and Vrieling, 2005; Doorduin and Vrieling, 2011). In fact, Japanese specimens of *R. okamurae* yielded a concentration of dilkamural of 1.9% (w/w) with respect to freeze-dried alga (Ninomiya et al., 1999), which is less than half of the mean concentration recorded in the present study of specimens from the Strait of Gibraltar, and even only a third of more recent measurements (dilkamural was 5.65 ± 0.16% of algal dry weight, data not shown). These data suggests that the content of dilkamural in its native range is markedly lower than in the non-native range (Strait of Gibraltar). Nonetheless, this comparison is preliminary since it would be necessary to perform updated quantitative analysis of dilkamural in fresh specimens of *R. okamure* from their native range (northwestern Pacific ocean) using the same biomass handling and analytical procedures that those employed for our invasive specimens. A second plausible explanation is that herbivore pressure during the first stages of the invasion may produce a positive selection toward those specimens (i.e. genotypes) with high concentrations of dilkamural, which later may potentially establish and spread faster (Blossey and Nötzold, 1995).

There are only few studies where specimens of invasive algae have been chemically analyzed, and the isolated compounds tested at their natural concentrations for anti-herbivory properties. In this regard, the cleavage products derived from dimethylsulfoniopropionate (DMSP), a metabolite present in the invasive *Codium fragile* spp. *tomentosoides*, demonstrated to be feeding deterrent against the sea urchin *Strongylocentrotus droebachiensis* (Lyons et al., 2007), while the invasive *Gracilaria vermiculophylla* was shown to produce, upon wounding or grazing, a series of arachidonic acid-derived oxylipins, among which the prostaglandin PGA2 and the hydroxylated fatty acid 7,8-di-HETE are deterrents for the herbivores *Idotea baltica* (Nylund et al., 2011) and *Echinolittorina peruviana* (Rempt et al., 2012), respectively. Moreover, the compound 1,1,3,3-tetrabromo-2-heptanone produced by the invasive red alga *Bonnemaisonia hamifera* was shown to possess feeding deterrent properties against various generalist mesograzers (Enge et al., 2012).

Although our results suggest that the existence of a novel chemical weapon could be involved in the escape of *R. okamurae* from native herbivores and that this may be a key mechanism contributing to explain the great invasive capacity of this species in the Strait of Gibraltar, other chemical strategies, including allelopathy or alteration of microfouling communities, may be also likely involved in the success of *R. okamurae*, as demonstrated for the exotic seaweed *Asparagopsis taxiformis* (Greff et al., 2017). Therefore, the possibility that the major compound (i.e. dilkamural) of *R. okamurae* provides additional advantages cannot be disregarded, and may also help to explain the outcompeting capacity of *R. okamurae* against the native sympatric species *D. dichotoma* (García-Gómez et al., 2020). In particular, the antimicrobial activity exhibited by dilkamural (Ninomiya et al., 1999) may suggest that this compound could also be effective against pathogen microorganisms present in the new habitats. Moreover, besides dilkamural, we have shown the presence in the invasive *R. okamurae* of other metabolites (compounds **2**–**6** in Fig. 1) that could also have a role in the defense, and hence in the success of the invasion by the alga, although their ecological role still needs to be further investigated.

In summary, we have demonstrated that the deterrent and toxic properties of the macroalga *R. okamurae* over a generalist herbivore (i.e. *P. lividus*) are mostly mediated by dilkamural, the major secondary metabolite produced by this invasive species. Since dilkamural has not been described in any alga of the area of the Strait of Gibraltar, the deterrent and toxic effects observed on the native generalist herbivore *P. lividus* are consistent with the novel weapons hypothesis (Callaway and Ridenour, 2004), which proposes that invaders benefit from the effects caused by their metabolites on native species which have never been exposed to these compounds. Moreover, the effect of dilkamural was concentration-dependent, displaying deterrent and toxic effects only under the concentration recorded in invasive specimens.

Authors statement

I. Casal-Porras: investigation, writing-review and editing; E. Zubía: conceptualization, investigation, writing- original draft, review and editing; F.G. Brun: conceptualization, investigation, funding acquisition, writing- original draft, review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2021.107398.

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